Summary

Gelsolin is a six domain Ca^{2+} sensitive protein central to actin dynamics, gelsolin exists in two forms a compact globular inactive shape under Ca^{2+} free conditions and physiological pH, which is activated to potential by 1 mM Ca^{2+} or low pH (Burtnick, Koepf et al. 1997; Ashish, Paine et al. 2007; Garg, Peddada et al. 2011). The blood plasma version of gelsolin forms an integral part of actin scavenging mechanism thereby preventing damage to the microvasculature by limiting the blood viscosity (Vasconcellos, Allen et al. 1994). Gelsolin is a well studied protein with crystal structure of full length inactive protein as well as crystal structures of activated halves bound to the natural substrate actin. However, to date no high resolution structure exists for gelsolin activated to potential, but a major understanding in this direction comes from our group where we showed the stepwise activation of gelsolin by highlighting the intermediate stages of activation and the role of linkers in extension and orientation of domains enabling actin binding (Ashish, Paine et al. 2007). Similarly the activation of gelsolin at low pH is also reported in literature but an understanding from the structural point of view remained limited (Lamb, Allen et al. 1993). Using SAXS data our group developed an insight towards the activation of the protein as a function of pH, where pH enables the opening of G1 domain as well as g1-g2 linker which is primarily responsible for the F-actin depolymerising activity (Garg, Peddada et al. 2011). These experiments as well as other experiments have shed light on the understanding of the structure and function of gelsolin, but a factor which is not directly addressed till now is, temperature. The majority of understanding about the function and structure of gelsolin comes from data collected at sub physiological temperatures. Thus, using the experience gained previously by our group in field of gelsolin and SAXS, this thesis was aimed at understanding the effect of temperature on the global shape and performance of gelsolin and its minimal version. Literature survey showed that earlier attempts were made to study the effect of temperature on gelsolin, however these effects mainly focused on the Ca^{2+} binding profiles and opening

of C-tail latch (Lin, Mejillano et al. 2000; Lueck, Yin et al. 2000). As a first step of this study the F-actin depolymerising activity of gelsolin heated at temperatures ranging from 10° to 80° C was measured. Gelsolin under Ca^{2+} and low pH activated conditions remained active up to 45° C after which it started losing its activity, but surprisingly there was display of activity under EGTA condition at temperatures around the physiological temperature. To probe if there were any changes in the shape of protein as an effect of temperature, SAXS data was collected by heating the protein from 10° to 80° C under three conditions in presence and absence of Ca^{2+} ions as well as at pH 5 without Ca^{2+}. The R_g was determined for each temperature and plotted as a function of temperature, the dimensions observed at 10° C were in accordance with the previously reported values and it could be clearly observed that the dimensions of the protein increase with increase in temperature. The R_g increases from 3.4 ± 0.23 nm at 10° C to 3.71 ± 0.29 nm at 40° C similarly the D_{max} also changes from 10.2 nm to 12 nm, an increase of ~20% in the length of the protein. However, the increase in dimensions due to increase in temperature was less than that observed for Ca^{2+} activated protein (12 nm and 15 nm, respectively), this indicated an incomplete opening of the molecule. This observation was made again with dummy atom modelling generated using the SAXS data, the SAXS models hence obtained were overlaid with the crystal structure for full length gelsolin, the model for gelsolin obtained at 10° C fitted well with the crystal structure, but in case of models obtained for 30° and 40° C unoccupied volume could be seen arising from the molecule. In absence of a crystal structure for gelsolin protein activated to potential the structures of activated halves of gelsolin were superimposed with the model manually and it was observed that the empty volume arising from the shape was actually G1 domain extending outwards. The semi open shape of gelsolin shape is maintained due to presence of the Ca^{2+} sensitive C-tail latch; if this inhibition is removed then temperature is capable for complete activation of gelsolin. Since it is already documented in literature that the extension of G1 arm is prerequisite for the depolymerization activity of gelsolin, thus even though the C-tail latch is closed but with

extended G1 domain and g1-g2 linker, gelsolin is capable for performing depolymerization of F-actin (Ashish, Paine et al. 2007; Garg, Peddada et al. 2011).

With gelsolin protein capable of performing depolymerization activity at physiological temperature, it may prove problematic at cellular levels as unchecked depolymerization of gelsolin may lead to blebbing of cell and ultimately apoptosis. However, this situation is avoided by the presence of PIP_2 – a phospholipid responsible for deactivation of gelsolin. SAXS data collected for gelsolin in presence of PIP_2, showed that PIP_2 maintains the closed shape of gelsolin after being heated at 40° C. Furthermore, if PIP_2 is added to pre-heated gelsolin protein, it is capable of reversing the partially opened gelsolin back to the compact inactive shape. Thus, it can be concluded that gelsolin protein is semi open and partially active at physiological temperature, but it is PIP_2 that maintains gelsolin in its inactive compact shape.

Gelsolin specifically plasma gelsolin is not only a prognostic marker but an equally valuable therapeutic protein also (Peddada, Sagar et al. 2012). Gelsolin replacement therapy (GRT) refers to the therapy where gelsolin is supplied exogenously to the body under conditions where gelsolin levels have reduced such as trauma or sepsis. GST has been extensively studied in case of murine models challenged with burn injuries and sepsis (Rothenbach, Dahl et al. 2004; Lee, Waxman et al. 2007; Peddada, Sagar et al. 2012). GRT increased the survivability of LPS challenged mice by 88%, where as in case of rats challenged with cutaneous burns gelsolin ameliorates the pathophysiology of inflammation post burn injury (Rothenbach, Dahl et al. 2004; Lee, Waxman et al. 2007). GRT in spite of being a very promising therapy had a huge bottle neck for translation, the dosage of exogenous gelsolin. The optimum dosage of gelsolin for mice was estimated to be ~8 mg protein per animal which would translate to ~28 g for a 70 kg human being, this highlighted the requirement of minimized but an efficient version of gelsolin to be used in GRT (Peddada, Sagar et al. 2013). Bearing this in mind our group rationally designed minimal versions of N-terminal of gelsolin, of all the variants fragment 28-161 was the most

promising candidate. Just like the parent molecule fragment 28-161 was capable of undertaking F-actin depolymerization under Ca^{2+} or low pH activated conditions only. Fragment 28-161 did not only depolymerise F-actin faster but performed better in vivo as compared to the full length protein. When fragment 28-161 was administered to LPS challenged mice, it was observed that fragment 28-161 performed three times better than full length protein despite being at four times less concentration than gelsolin (Peddada, Sagar et al. 2013). Thus, fragment 28-161 became a favourable candidate to study the effect of temperature.

When heated fragment 28-161 was used for F-actin depolymerization, it was observed that protein was active under Ca^{2+} and pH activated conditions only but not under EGTA conditions. Interestingly, the protein was able to maintain its activity even after being heated at 80° C, this kind of property was not observed in case of the full length gelsolin, indicating the thermostable nature of fragment 28-161. When CD data was collected for fragment 28-161 it was observed that there was increase in the negative intensity of the protein with increase in temperature suggesting a gain in secondary structure of the protein as a function of temperature. This was observed in case of protein heated in presence as well as absence of Ca^{2+} ions; furthermore, this change in ellipticity was influenced by the concentration of protein used for the study. When high concentration of fragment 28-161 was heated in absence of Ca^{2+} ions it was observed that there was increase in intensity, but in presence of Ca^{2+} the CD profile showed two curves. First there was increase in the negative intensity up to 70° C after which there was a sudden decrease in negative intensity till it approached 0. From the CD curve it was clear that there is increase in the secondary structure content of protein but it was not clear if this was due to intermolecular or intramolecular processes.

SAXS data was collected for fragment 28-161 heated from 20 to 80° C, where just like the parent molecule there was increase in dimensions of the protein as a function of temperature. The dimensions recorded for fragment 28-161 were found to be higher

than those of reported earlier. Even the models generated from the SAXS data could fit nearly two monomers abstracted from the structure of inactive molecule; interestingly, same observation was made for data collected in presence of 1 mM Ca^{2+} this data indicated intermolecular processes are probably responsible for the dimer formation. This was quite puzzling for us as the protein used for data collection was FPLC purified and only the peak observed at monomer position was used for SAXS data collection.

FPLC experiments done with high and low concentration of protein showed that with increase in concentration protein showed dimerization. On the other hand, if protein at low concentration was heated from 10° to 50° C, oligomerization was observed this lead to a question what drives oligomerization concentration, temperature or both. This was answered by set of experiments where FPLC was performed using various concentration of protein heated form 10° to 50° C and monomer fraction was estimated for each case. It was observed that with increase in temperature the protein underwent oligomerization; this was faster as the concentration increased. The change in oligomeric state of protein under 1mM Ca^{2+} was similar to that observed in case of 0 mM Ca^{2+} but the shift was slightly delayed. Hence it was concluded that it was both temperature and concentration that drive the oligomerization of protein.

The crystal structure for fragment 28-161 was obtained under Ca^{2+} bound condition, with two protein chains in an asymmetric unit interacting each other by series of non-covalent interactions along the helices. The various non-covalent interactions that hold the protein together include a H-bond and salt bridge, apart from these two ionic interactions there are fourteen non-bonded contacts along the helices. Upon comparing the structure of fragment 28-161 to that of inactive and active gelsolin, it was observed that the domain of fragment 28-161 retains its basic gelsolin domain architecture, but the g1-g2 linker is oriented as in the active structure. Furthermore, when the dimer of fragment 28-161 was compared with the crystal structure of N-terminal half of gelsolin bound to actin it was observed that the molecules arrange themselves such that each monomer mimics

as actin molecule to the other monomer. The residues responsible for the interactions that held the molecules together were also involved in interaction with actin. The crystal structure obtained in this study was in well accordance with the solution shape observed for the SAXS collected in this study.

Fragment 28-161, the minimal version is a thermostable version of gelsolin, the probable reason behind this thermostability can be attributed to the oligomer formation of the protein. Furthermore, this oligomer that is formed has capacity to dissociate in to functional monomer in presence of Ca^{2+} and actin the natural substrate, hence it maintains its F-actin depolymerization activity even after being heated at 80° C. Attempts were made to enhance thermostability of 28-161 through rational site directed mutagenesis, this enhancement in thermostability was aimed with no loss in activity or actin and Ca2+ binding capacity of the protein. Mutant proteins were expressed and assayed for F-actin depolymerization. It was observed that as a result of mutagenesis the activity of fragment 28-161 was compromised, this suggested that the sequence of wild type is naturally optimized for thermostability and hence the mutagenesis of protein was discontinued.

Overall this thesis deals with the study of effect of temperature on the global shape of gelsolin, which has so far remained somewhat neglected factor. The results have shown that gelsolin at physiological temperature remains in a semi open condition hence remaining partially active, but this is avoided due to presence of PIP_2 in the cell that maintains the closed shape. Fragment 28-161, the promising biobetter of gelsolin also possess the property of thermostability, also the crystal structure obtained for fragment 28-161 is first report where a truncate of active N-terminal gelsolin is obtained but in absence of its natural substrate actin.